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Ion transport by rabbit nonciliated bronchiolar epithelial cells (Clara cells) in culture

(ouabain/amiloride/bumetanide/isoproterenol)

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ABSTRACT **The functions of epithelia that line small airways in mammalian lungs are unknown. To gain insight into the role of small-airway epithelia in lung liquid balance, Clara cells were isolated from excised rabbit lungs by enzymatic digestion, enriched by centrifugal elutriation and density centrifugation, and further purified by differential adherence to collagen matrices. The resulting cell population was composed of 85% Clara cells, 3% ciliated cells, and <1% macrophages. The remainder of the cells were not definitively identified. The transepithelial potential difference peaked on day 3 in culture. Preparations studied in Ussing flux chambers exhibited a potential difference of 8 mV (apical bath negative), a resistance of 500 ohm·cm^2, and an equivalent short-circuit current (I_{SCeq}) of 16 μA/cm^2. Inhibition of the Na^+/K^+-ATPase by ouabain abolished I_{SCeq}. Exposure of the apical surface to amiloride or replacement of Na^+ in the apical bathing solution with an impermeant cation (*N*-methyl-D-glucamine) decreased I_{SCeq} by 66% and 93%, respectively. Neither amiloride in the basolateral bathing solution, nor bumetanide, nor isoproterenol significantly altered basal I_{SCeq}. These findings indicate that (*i*) Clara cells in culture form polarized monolayers, (*ii*) Clara cells transport Na^+ from the apical to the basolateral bathing solution, and (*iii*) the small airways of the rabbit may function in liquid absorption.**

Epithelia in proximal airways have been studied extensively, but little is known about epithelial function in the distal, or "small" (diameter <2 mm), airways. The small airways are located at the interface between alveoli and conducting airways and, therefore, occupy a strategic position from which to influence lung function. With respect to the overall balance of pulmonary liquids, the small airways could serve to generate liquids that move up airway surfaces or, conversely, serve to absorb liquid that moves off the large alveolar surface onto the smaller airway surface (1). Similarly, small-airway epithelia could serve to either scavenge surfactant moving off of the alveolar surface or secrete surfactant to prevent small-airway collapse (2–5). The importance of small-airway epithelia in maintenance of normal lung function is emphasized by the observation that dysfunction of the epithelia in this region is often the first manifestation of airway disease (6).

One approach to describe the functions of an epithelium is to study cell types unique to the region (7, 8). With respect to mammalian small airways, the Clara cell appears to be prevalent and relatively unique (9). Substantial indirect evidence from morphologic studies suggests that these cells modify the liquid that lines normal small airways. Clara cells of most species contain abundant agranular endoplasmic reticulum (AER) and membrane-bound granules. Histochemical and EM studies have revealed that the granules contain protein that may be secreted onto the airway surface (5, 10, 11). Other morphologic evidence has indicated that Clara cells synthesize lipids that are released into the fluid lining the bronchioles (5). The presence of extensive folds in the basolateral membrane of Clara cells has led to speculation that these cells transport salt and water and thereby regulate fluid balance in the small airways (12, 13).

The major limitations to the detailed study of Clara cell function have been the small diameter and inaccessibility of the bronchioles. In the present study, ion-transport functions of Clara cells were evaluated using cell culture techniques.

MATERIALS AND METHODS

Cell Isolation. Clara cells were isolated from male New Zealand White rabbits (1.5–3 kg) by techniques described in detail by Devereux and Fouts (14). Rabbit tracheal epithelial cells were isolated from excised airways by enzymatic digestion [0.1% protease type XIV (Sigma) plus 0.01% DNase (Sigma)] in Eagle's minimal essential medium (MEM) at 4°C for 20–24 hr. Fetal bovine serum (FBS; 10%, vol/vol) was added to stop digestion and the disaggregated cells were collected by filtration through Nitex (60-μm mesh) and centrifugation (500 × *g*, 4°C). The cells were washed in MEM/10% FBS, and resuspended (5 × 10^6 viable cells per ml) in Ham's nutrient mixture F-12 supplemented with FBS (5%, vol/vol), gentamicin (50 μg/ml), penicillin (50 units/ml), and streptomycin (50 μg/ml).

Cell viability was determined by trypan blue exclusion. Clara cells were identified by staining with nitroblue tetrazolium (NBT) (14). Type II cells were identified by using a fluorescent dye, phosphine 3R (8). Cells were also identified by EM. Thin sections (60–80 nm) were prepared by fixing preparations with 2.5% glutaraldehyde in 100 mM phosphate buffer (pH 7.4), postfixing in osmium, and embedding in Epon. Thin sections were viewed with a Zeiss electron microscope (model 10A, Oberkechen, F.R.G.).

Cell Culture. The construction of collagen matrix supports (CMS) has been described (15). In brief, crosslinked-collagen matrices were attached to polycarbonate supports (orifice diameter, 0.4 cm), sterilized in a solution of ethanol (70%, vol/vol) in distilled water, and equilibrated with F-12. Rabbit tracheal epithelial cells were seeded onto the CMS (10^6 cells per cm^2) and allowed to attach overnight. The adherent cells were maintained in F-12/5% FBS in 5% CO_2/95% air at 99% humidity and 36.5°C. When the cells were confluent, they were removed by incubation with 20 mM EDTA (37°C, pH 7.4, 2–24 hr). Clara cells (10^6 NBT-positive cells per cm^2) were seeded onto CMS preconditioned by rabbit tracheal epithelial cells and cultured as described above.

Thymidine Labeling. [*methyl*-^{3}H]Thymidine and nonlabeled thymidine were added to F-12/5% FBS to achieve a

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Abbreviations: CMS, collagen matrix support(s); I_{SCeq}, equivalent short-circuit current; AER, agranular endoplasmic reticulum; FBS, fetal bovine serum; NBT, nitroblue tetrazolium.

specific activity of 1.25 μCi/ml (1 Ci = 37 GBq) and a thymidine concentration of 5 μM. ^{3}H-containing medium was added to the apical compartment of CMS preparations that had been in culture for 20 hr. Either 6 or 48 hr later, the medium was removed, the CMS was rinsed, and the cells were fed with nonradioactive medium. The cultures were fed daily until the fourth day, when they were were air-dried and overlaid with liquid photographic emulsion (Kodak NTB-3). After a 3-day exposure period, the emulsion was developed (Kodak Microdol). A minimum of 500 cells from each preparation were scored by light microscopy.

Bioelectric Measurements. The spontaneous transepithelial potential difference was measured daily, using calomel half-cells (Radiometer, Copenhagen) connected to a high-impedance voltmeter (World Precision Instruments, New Haven, CT). To measure the resistance, the CMS were mounted in a modified Ussing flux chamber. The apical and basal surfaces were perfused continuously at a rate of 1 ml/min with F-12/5% FBS. In some experiments, tissues were perfused with Krebs–Ringer bicarbonate (KR) solution (115 mM NaCl/25 mM $NaHCO_3$/2.4 mM K_2HPO_4/0.4 mM KH_2PO_4/1.2 mM $CaCl_2$/1.2 mM $MgCl_2$/10 mM glucose/5% FBS) and KR solution in which *N*-methyl-D-glucamine was substituted for Na^+. Current pulses (15–50 μA/cm^2, 500-msec duration, 5-sec interval) were passed across the tissue by means of 3 M KCl/agar bridges. The resistance was calculated from the magnitude of the current pulse and the change in potential difference, using Ohm's law, and the equivalent short-circuit current (I_{SCeq}) was calculated from the resistance and the spontaneous potential difference.

Response to Pharmacologic Agonists. Responses to drugs were assessed on preparations mounted in the modified Ussing flux chamber. Drugs were applied to the apical or basolateral bathing solutions via the perfusion system. The tissues were exposed to the drugs until steady-state responses were achieved. Ouabain (Sigma), bumetanide (Leo Pharmaceuticals, Copenhagen), and isoproterenol (Sigma) were used at concentrations of 0.1 mM, 0.1 mM, and 0.01 mM, respectively. Amiloride (Merck Sharp & Dohme) was used at a concentration of 0.1 or 1 mM.

Statistics. The values reported are means ± SEM for *n* different isolations. The statistical significance of changes induced by drugs or ion substitutions was tested by comparing the changes induced by each maneuver with the time control group, using an unpaired *t* test ($P < 0.05$).

RESULTS

Approximately 3×10^6 cells per rabbit (>90% viability) were harvested from the Clara cell band in the density gradients. Fifty-five ± 2.9% ($n = 17$) of the cells stained darkly with NBT and, therefore, were considered to be Clara cells. Type II and ciliated cells accounted for 7 ± 2% ($n = 5$) and 6 ± 2% ($n = 6$), respectively. The rest were identified as alveolar macrophages and plasma cells by transmission EM.

All of the cells from the Clara cell fraction that attached to preconditioned CMS within 4 hr after seeding stained darkly with NBT. Rabbit tracheal epithelial cells in culture for 4 hr stained lightly with NBT. After 16 hr in culture both tracheal and Clara cells stained darkly, indicating that nonspecific staining developed in culture. Less than 1% of the cells attached to the CMS after 4 hr stained with phosphine 3R. No ciliated cells were identified in 4-hr cultures by phase-contrast microscopy. These observations indicated that Clara cells preferentially attached to the CMS during the first 4 hr in culture.

Light microscopic examination of all preparations revealed that confluence was generally achieved within 16 hr in culture. To determine the cellular composition of the confluent sheets, four preparations were fixed 16 hr after seeding, and 32 ± 4 cells were examined from each preparation by EM. Eighty-five ± 2% of the cells examined could be definitively identified as Clara cells by the presence of electron-dense secretory granules, abundant AER, and/or two distinct types of mitochondria (rod-shaped, which contained cristae, and round, which lacked cristae and were filled with a granular matrix) (Fig. 1 *A–C*). Eleven ± 1% of the cells appeared similar to Clara cells in terms of general shape, nucleus to cytoplasm ratio, and density of the cytoplasm but did not meet the above specific criteria. These cells often formed tight junctions with adjacent morphologically identifiable Clara cells and did not contain lamellar bodies or abundant rough endoplasmic reticulum. Therefore, these cells could not be identified as basal cells, alveolar type II cells, or plasma cells. Three ± 1.2% were ciliated, and one macrophage was identified.

Cells on the CMS 4 days after seeding contained AER and two distinct types of mitochondria. However, few electron-dense granules were observed (Fig. 1*D*). Ciliated cells were observed rarely (<1%), and no macrophages were observed.

Four cultures were exposed to [^{3}H]thymidine to determine if the cells were actively dividing. ^{3}H was incorporated into <1% of the nuclei in each culture. Lengthening the duration of exposure from 6 hr to 48 hr did not increase the number of labeled nuclei. These data suggested that neither the Clara cells nor unidentifiable cells were proliferating during the first 4 days in culture.

The monolayers generated a potential difference that peaked between the second and fourth days after seeding (Fig. 2). A wide range of peak potentials was recorded (1–27 mV), but no relationship could be established between the potential difference generated by the preparations and the purity of the cell suspension from which the monolayers were derived (correlation coefficient $r = 0.25$, $n = 20$). These data indicate that a contaminating cell type probably was not responsible for the variation in the spontaneous potential difference.

Bioelectric properties were characterized more completely in preparations from 23 isolations at or within 1 day of the development of the peak potential difference. For these preparations, the potential difference was 8.3 ± 2.0 mV (apical bath negative). Current–voltage plots (−40 to +40 mV) were linear, allowing calculation of the resistance by Ohm's law. The resistance was calculated to be 500 ± 71 ohm·cm^2, and I_{SCeq} was 15.9 ± 2.0 μA/cm^2. To ensure that I_{SCeq} was an accurate estimate of the "true" short-circuit current (I_{SC}), a few preparations were clamped to zero potential difference and the imposed current was measured. I_{SC} was found to be identical to I_{SCeq}. The spontaneous potential difference was directly related to the resistance and correlated poorly with I_{SCeq} (Fig. 3).

Ouabain, which inhibits the Na^+/K^+-ATPase, abolished I_{SCeq} across the cultured monolayers. Because the Na^+/K^+-ATPase has been shown to fuel both Na^+ absorption (16) and/or Cl^- secretion (17) in other respiratory epithelia, inhibitor and ion-substitution experiments were performed to further characterize the transepithelial current flow. Amiloride (0.1 mM), which blocks passive Na^+ conduction, reduced I_{SCeq} by 66 ± 4% ($n = 8$) and increased the resistance R by 19 ± 8% ($n = 8$) when added to the apical bathing solution (Table 1). Increasing the concentration of amiloride to 1 mM did not result in greater inhibition. In contrast, amiloride had no effect when added to the basolateral bathing solution ($\Delta I_{SCeq} = 6 \pm 6\%$, $\Delta R = 7 \pm 9\%$, $n = 4$). These responses are consistent with transepithelial Na^+ absorption involving passive Na^+ influx across the apical cell membrane and active extrusion of Na^+ across the basolateral cell membrane.

The relationship of the Na^+ concentration of the bathing solution and the current flow across the Clara cell monolay-

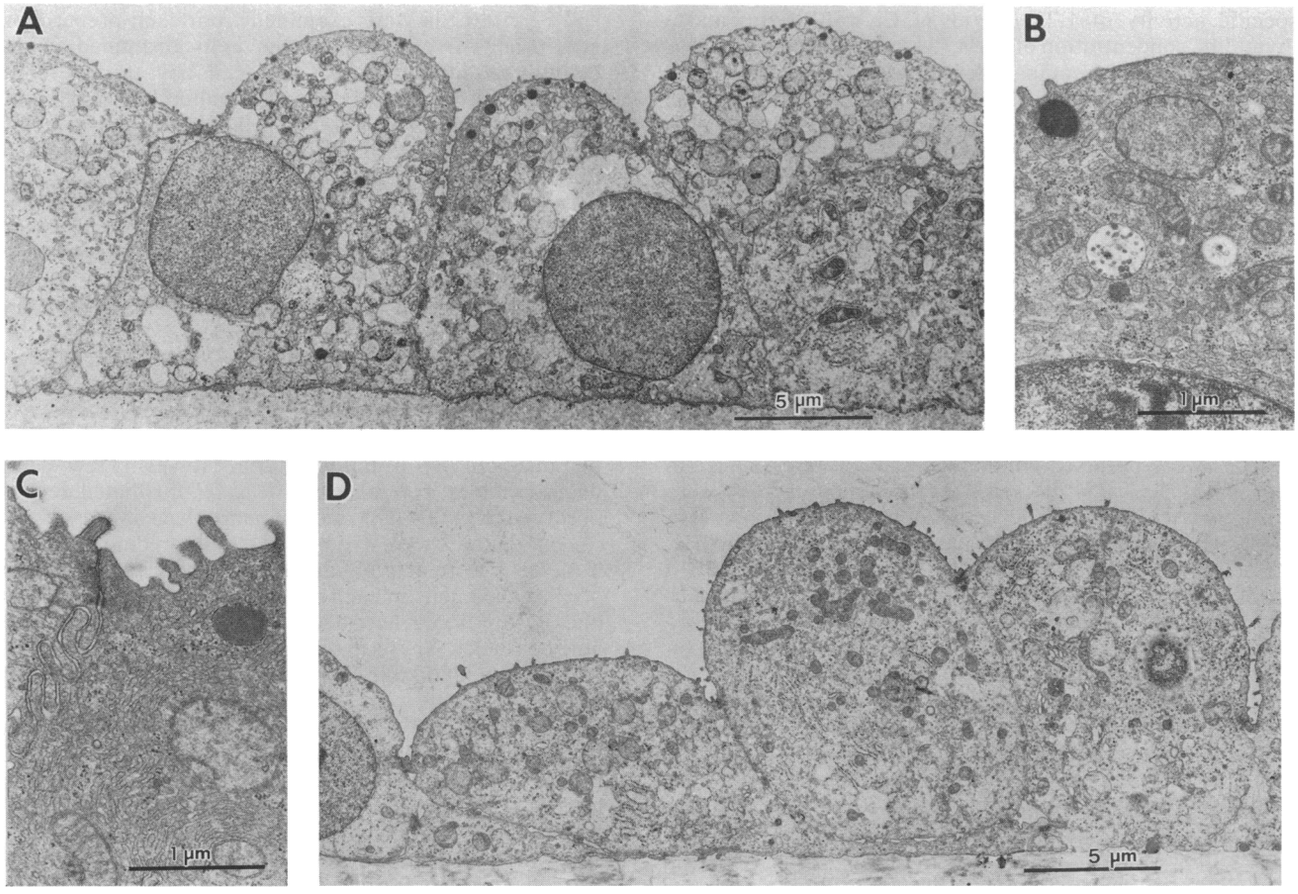

FIG. 1. Transmission electron micrographs of cells from the Clara cell fraction after 16 hr (*A–C*) and 96 hr (*D*) in culture. AER (*A–D*), large mitochondria without cristae (*B* and *C*), electron-dense secretory granules (*A–C*), and tight junctions (*C*) were observed in 16-hr cultures. After 4 days in culture, few electron-dense granules were observed (*D*).

ers was also examined by substituting an impermeant cation, *N*-methyl-D-glucamine, for Na^+. Reduction of the Na^+ concentration of the apical bathing solution from 140 mM to 7.5 mM reduced I_{SCeq} by 93 ± 16% and increased the resistance R by 28 ± 10% (n = 4). Unilateral reduction of Na^+ concentration in the basolateral solution to 7.5 mM tended to increase both I_{SCeq} and R (ΔI_{SCeq} = 30 ± 14%, ΔR = 43 ± 25%, n = 4). The larger decrease in I_{SCeq} with reduction of the Na^+ concentration in the apical bath as compared to the decrease with amiloride may reflect (*i*) more complete inhibition of the cellular path for Na^+ movement and/or (*ii*) passive ion currents induced by asymmetrical solution Na^+ concentrations.

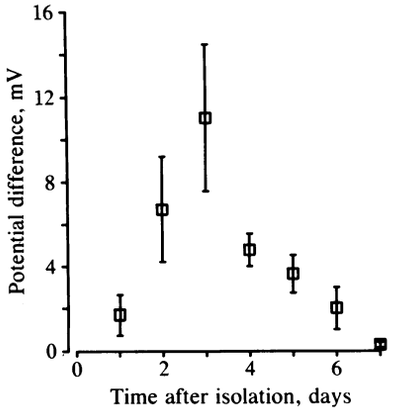

FIG. 2. Time course of the spontaneous transepithelial potential difference generated by Clara cell monolayers in culture. Values represent means ± SEM for 11 CMS preparations from eight different isolations.

Bumetanide, a loop diuretic that inhibits Cl^- secretion by canine tracheal epithelia (18), had no significant effect on the tissues in the absence of amiloride when added to the basolateral (Table 1) or apical solutions (ΔI_{SCeq} = 7 ± 6%, n = 3). However, when added to the basolateral bathing solution of preparations that had been pretreated with amiloride, bumetanide induced a 20 ± 7% (n = 3) decrease in I_{SCeq}. These observations are consistent with the notion that the current generated by unstimulated Clara cells is predominantly due to amiloride-sensitive Na^+ absorption, and the cells have the capacity to secrete a small amount of Cl^- when Na^+ entry into the cell is inhibited. Isoproterenol, a drug that stimulates Cl^- secretion across proximal airways but stimulates Na^+ absorption by rat alveolar type II cells (19), had no effect on resistance or I_{SCeq} (Table 1).

DISCUSSION

The validity of using cultured cells to estimate the *in situ* function of pulmonary epithelia rests upon evidence that current cell-culture techniques can yield preparations with functional characteristics resembling those of intact tissues (15, 20–24). Application of these techniques to estimate the *in situ* function of small-airway epithelia requires that a relatively pure population of cells can be obtained from this region and maintained in culture. Furthermore, functional

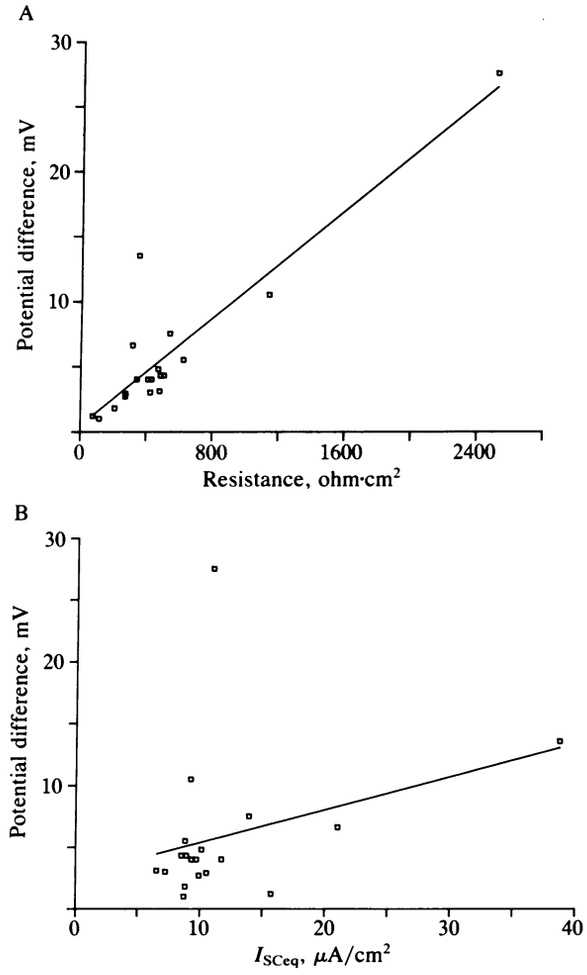

FIG. 3. Spontaneous potential difference vs. resistance (*A*) and I_{SCeq} (*B*). Measurements were made on the fourth day in culture. Correlation coefficients (*r*) were 0.9045 (*A*) and 0.3174 (*B*).

aspects of the isolated cell population must represent a dominant activity in the small-airway epithelia.

Several groups have demonstrated that pulmonary epithelial cells in culture form barriers with ion-transport characteristics qualitatively similar to those of the freshly excised tissues from which the cells were obtained. Coleman *et al.* (20) and Welsh (21) reported that cultured canine tracheal epithelial cells retained their ability to absorb Na^+ in the basal state and secrete Cl^- when stimulated with β-adrenergic agonists. Boucher *et al.* (22) showed that canine tracheal and bronchial epithelial cells retain their respective Cl^--secretory and Na^+-absorptive functions in primary culture. Yankaskas *et al.* (15) and Widdicombe *et al.* (23, 24) reported that transport properties of epithelial cells from the respiratory tract of normal and cystic fibrosis patients are qualitatively preserved in culture. These findings indicate that transport characteristics of epithelial cells from proximal airways are preserved in culture.

Clara cells were isolated and partially purified by protease digestion of rabbit lungs followed by centrifugal elutriation and Percoll density centrifugation of the cell digest. Approximately 15% of the cells in the enriched Clara cell fraction were ciliated or alveolar type II cells. However, the cells attached to the CMS after 4 hr in culture appeared to be Clara cells, and not type II or ciliated cells, on the basis of NBT staining, phosphine 3R staining, and phase-contrast microscopy. These observations indicated that Clara cells preferentially attached to the preconditioned CMS.

Due to the increase in nonspecific NBT staining with time in culture, EM criteria were used to evaluate the cellular composition of the monolayers. Plopper *et al.* (13) reported that rabbit Clara cells *in vivo* can be identified by the presence of an apical dome, electron-dense secretory granules, and abundant AER. In addition, these cells contain two types of mitochondria: one type is rod-shaped and contains numerous cristae; the other is round (2-μm diameter), lacks cristae, and is filled with a granular matrix (13). On the basis of these morphologic criteria, 85% of the cells in culture after 16 hr were definitively identified as Clara cells. Approximately 3% of the cells could be identified as ciliated cells, and no alveolar type II cells were observed. These data also indicate that Clara cells preferentially attach to the CMS.

Eleven percent of the cells could not be definitively identified after 16 hr in culture. They did form tight junctions, indicating that they were epithelial in nature. Therefore, these cells may have been type II and/or ciliated cells that underwent morphologic dedifferentiation during the first 16 hr in culture. However, alveolar type II cells have been shown to retain their morphology (i.e., lamellar bodies) for 20 hr in culture (8). No lamellar bodies were observed in these preparations when examined by light or electron microscopy. Furthermore, preliminary work in our laboratory indicates that type II cells from humans and rabbits, as well as ciliated cells from humans, rabbits, and dogs, do not readily attach to collagen matrices. The few ciliated cells observed in cultures of mixed airway epithelial cells derived from rabbit trachea have retained their cilia for up to 2 weeks. Therefore, we do not believe that the unidentified cells on the CMS were type II cells or ciliated cells that had dedifferentiated. A more plausible explanation stems from work by Evans *et al.* (25) in which three morphologically distinct categories of Clara cells were described. Type A Clara cells are similar to the cells described by Clara but have variable amounts of AER and do not contain electron-dense granules. The unidentified cells within our monolayers may have been type A Clara cells.

Table 1. Response of Clara cell monolayers to mediators of ion transport

		Before		During	
Condition	*n*	I_{SCeq}, μA/cm²	Resistance, ohm·cm²	I_{SCeq} μA/cm²	Resistance, ohm·cm²
Control	13	12.8 ± 1.5	468 ± 167	10.7 ± 0.9	489 ± 172
Ouabain	4	8.7 ± 2.5	647 ± 263	1.0 ± 0.6*	636 ± 278
Amiloride	8	14.1 ± 3.0	554 ± 161	5.3 ± 1.5*	731 ± 262*
Bumetanide	5	11.3 ± 1.5	342 ± 75	11.4 ± 1.6	344 ± 75
Isoproterenol	6	8.6 ± 0.7	534 ± 161	7.2 ± 0.9	439 ± 60

Clara cells cultured on CMS for 4 days were mounted in a modified Ussing flux chamber and perfused with Ham's nutrient mixture F-12/5% FBS. Drugs were administered through the perfusion system. Amiloride (0.1 mM) was applied to the apical surface. Ouabain (0.1 mM), bumetanide (0.1 mM), and isoproterenol (0.01 mM) were applied to the basolateral surface. Values represent mean ± SEM.
*Significantly different from control values ($P < 0.05$).

Specific markers for these cells will be required to test this hypothesis.

Few electron-dense secretory granules were observed by day 4, but AER and two different types of mitochondria were still present. In addition, experiments using [^{3}H]thymidine indicated that minimal mitotic activity occurred between days 1 and 4. Therefore, the population of cells on the CMS after 4 days appeared to be the same population that originally attached to the matrix.

Resistance and I_{SCeq} of the monolayers were variable (Fig. 3), but bioelectric parameters were not related to the purity of the suspensions used to seed the CMS. Furthermore, preparations that displayed extreme values were morphologically similar by light and electron microscopic criteria to preparations exhibiting average potential differences and resistances. Therefore, it is unlikely that the variation in resistance and I_{SCeq} was due to a cell type other than the Clara cell.

The large decreases in I_{SCeq} following exposure of the apical surface of the Clara cell to amiloride or reduction of the Na^+ concentration of the apical bathing solution indicate that Na^+ absorption is the major active ion-transport system of the Clara cell. In the baseline state, bumetanide or Na^+ absorption is the major active ion-transport system of the Clara cell. In the baseline state, bumetanide or Na^+ replacement in the basolateral bathing solution did not inhibit I_{SCeq}, indicating that Clara cells display little spontaneous Cl^- secretion. However, the presence of a residual current after amiloride exposure may indicate that Cl^- secretion can be induced in Clara cells. The sensitivity of a portion of the residual current to bumetanide is consistent with this idea. Induction of Cl^- secretion by amiloride has been observed in human nasal (26) and bronchial (27) epithelia as well as rabbit trachea (16). The lack of a response to isoproterenol would suggest that neither Cl^- secretion or an acceleration of Na^+ absorption can be induced in the Clara cell preparation by β-adrenergic agonists.

The ion-transport properties of Clara cell monolayers appear to differ from those of epithelia from proximal airways or alveolar regions. The short-circuit current measured across freshly excised rabbit trachea is relatively large [$\approx$85 $\mu A/cm^2$ (16, 28)]. This current is only inhibited 40% by exposure to amiloride (16, 28) because a large furosemide-sensitive Cl^- secretion is induced by the drug. Similar results have been observed with primary cultures of rabbit tracheocytes (29). No data on the transport characteristics of rabbit alveolar epithelial cells are available, but sheets of cultured rat alveolar type II cells exhibit a small I_{SCeq} ($\approx$1 $\mu A/cm^2$) that is abolished by exposure to amiloride. β-Adrenergic agonists accelerate Na^+ absorption across the cultured alveolar type II cells (19). Therefore, it appears that the Clara cell may absorb Na^+ at a rate per unit area (i.e., I_{SCeq}/cm^2) that is intermediate between large-airway epithelial cells and alveolar type II cells but retains the capacity to secrete Cl^- typical of airway epithelia. Given the large aggregate surface area of the small airways and the large proportion of Clara cells in the small-airway epithelia of some mammals, the magnitude of Na^+ absorption contributed to the mammalian airway epithelium by the Clara cell may be relatively large.

We cannot establish that Clara cell function dominates ion-transport activity in the small airways. However, Clara cells account for $\approx$65% of the bronchiolar epithelial cells in rabbits and, consequently, would be expected to have a major influence on the net ion transport across small airways. The observation that the major ion translocation of this cell type is Na^+ absorption makes it likely that the small airways participate in volume absorption and are not the source of airway surface liquids that are moved proximally by ciliary motion. This estimate of overall function of the intact small-airway epithelia is buttressed by preliminary experiments with monolayers generated from mixed cell populations from canine small-airway epithelial cells. These preparations also appear to be absorptive in nature (unpublished observation).

In conclusion, we have developed a cell culture model that has been used to study ion-transport properties of epithelial sheets composed predominantly of Clara cells. Data derived from this model indicate that the Clara cells that line the distal airways of rabbits absorb Na^+. If counterions and water passively follow the flow of Na^+, Clara cells would promote fluid absorption from small-airway lumens. The culture model should also be useful for investigating other aspects of Clara cell function. The arrangement of the cells in a polarized monolayer is conducive to studies with labeled precursors similar to those previously performed *in vivo* (30), and the presence of electron-dense granules in the cultured cells may enable investigators to define the secretory products and processes of this cell type.

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